LUMI-CELL® ER ASSAY

AGONIST PROTOCOL

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

Developed by:

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LIST OF ACRONYMS AND ABBREVIATIONS

13 mm test tube	13 x 100 mm glass test tubes
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl Sulfoxide
DMSO control	1% v/v dilution of DMSO in tissue culture media used as a vehicle control
E2	17β-estradiol
E2 reference standard	11 Point Serial Dilution of 17β-estradiol reference standard for the LUMI-CELL® ER agonist assay
EC_{50} value	Concentration that produces a half-maximal response as calculated using the four parameter Hill function.
ER	Estrogen Receptor
Estrogen-free DMEM	DMEM (phenol red free) supplemented with 1% Penicillin/Streptomycin, 2% L-Glutamine, and 5% Charcoal-dextran treated FBS
FBS	Fetal Bovine Serum
G418	Gentamycin
Methoxychlor	*p,p'*-Methoxychlor
Methoxychlor control	3.13 μg/mL Methoxychlor Weak Positive Control for the LUMI-CELL® ER Agonist Assay
RPMI	RPMI 1640 growth medium
TA	Transcriptional Activation
T25	25 cm^2 tissue culture flask
T75	75 cm^2 tissue culture flask
T150	150 cm^2 tissue culture flask

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1.0 PURPOSE

This protocol is designed to evaluate coded test substances for potential estrogen receptor (ER) agonist activity using the LUMI-CELL® ER assay.

2.0 SPONSOR

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2.1 Substance Inventory and Distribution Management

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3.0 DEFINITIONS

- **Dosing Solution:** The test substance, control substance, or reference standard solution, which is to be placed into the tissue culture wells for experimentation.
- **Raw Data:** Raw data includes information that has been collected but not formatted or analyzed, and consists of the following:
    - Data recorded in the Study Notebook
    - Computer printout of initial luminometer data
    - Other data collected as part of GLP compliance, e.g.:
        - Equipment logs and calibration records
        - Test substance and tissue culture media preparation logs
        - Cryogenic freezer inventory logs
- **Soluble:** Test substance exists in a clear solution without visible cloudiness or precipitate.
- **Study Notebook:** The study notebook contains recordings of all activities related to the conduct of the LUMI-CELL® ER agonist assay.
- **Test Substances:** Substances supplied to the testing laboratories that are coded and distributed such that only the Project Officer, Study Management Team (SMT), and the Substance Inventory and Distribution Management have knowledge of their true identity. The test substances will be purchased, aliquoted,

coded, and distributed by the Supplier under the guidance of the NIEHS/NTP Project Officer and the SMT.

4.0 TESTING FACILITY AND KEY PERSONNEL[1]

4.1 Testing Facility

Xenobiotic Detection Systems, Inc. (XDS), 1601 E. Geer St., Suite S, Durham, NC 27704

4.2 Key Personnel

- Study Director: John Gordon, Ph.D.
- Quality Assurance Director: Mr. Andrew

5.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

5.1 Test Substances

Test substances are coded and will be provided to participating laboratories by the Substance Inventory and Distribution Management team.

5.2 Controls

Controls for the ER agonist protocol are as follows:

Vehicle control (dimethyl sulfoxide [DMSO]): 1% (v/v) DMSO (CASRN 67-68-5) diluted in tissue culture media.

Reference standard (17β-estradiol [E2]): Three concentrations of E2 (CASRN 50-28-2) in duplicate for range finder testing and a serial dilution consisting of 11 concentrations of E2 in duplicate for comprehensive testing

Positive control (p,p'-Methoxychlor [methoxychlor]): Methoxychlor (CASRN 72-43-5), 3.13 µg/mL in tissue culture media, used as a weak positive control.

[1] Testing facility and personnel information are provided as an example.

6.0 OVERVIEW OF GENERAL PROCEDURES FOR AGONIST TESTING

All experimental procedures are to be carried out under aseptic conditions and all solutions, glassware, plastic ware, pipettes, etc., shall be sterile. All methods and procedures shall be documented in the study notebook.

Agonist range finder testing is conducted on 96-well plates using four concentrations of E2 (5.00×10^{-5}, 1.25×10^{-5}, 3.13×10^{-6} and 7.83×10^{-7} μg/mL) in duplicate as the reference standard and four replicate wells for the DMSO control. Range finder testing uses all wells of the 96-well plate to test six substances as seven point 1:10 serial dilutions in duplicate.

Comprehensive testing is conducted on 96-well plates using 11 concentrations of E2 in duplicate as the reference standard (**Table 6-1**). Four replicate wells for the DMSO control and four replicate wells for the methoxychlor control are included on each plate. Comprehensive testing uses all wells of the 96-well plate to test 2 substances as 11 point serial dilutions in triplicate.

Table 6-1 Concentrations of E2 Reference Standard Used in Comprehensive Testing

E2 Concentrations[1]		
1.00×10^{-4}	6.25×10^{-6}	3.92×10^{-7}
5.00×10^{-5}	3.13×10^{-6}	1.95×10^{-7}
2.50×10^{-5}	1.56×10^{-6}	9.78×10^{-8}
1.25×10^{-5}	7.83×10^{-7}	

[1]Concentrations are presented in μg/mL.

Visual observations for cell viability are conducted for all experimental plates just prior to luminescence measurements, as outlined in **Section 11.2**.

Luminescence data, measured in relative light units (RLUs), is corrected for background luminescence by subtracting the mean RLU value of the vehicle control (DMSO) wells from the RLU measurements for each of the other wells of the 96-well plate. Data is then transferred into Excel® data management spreadsheets and GraphPad PRISM® 4.0 statistical software, graphed, and evaluated as follows:

- A response is considered positive for agonist activity when the average adjusted RLU for a given concentration is greater than the mean RLU value plus three times the standard deviation for the vehicle control.

- Any response below this threshold is considered negative for agonist activity.

For substances that are positive at one or more concentrations, the concentration that causes a half-maximal response (EC_{50}) is calculated using a Hill function analysis. The Hill function is a four-parameter logistic mathematical model relating the substance concentration to the response (typically following a sigmoidal curve) using the equation below:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC50 - X)\text{HillSlope}}}$$

where Y = response (i.e., relative light units); X = the logarithm of concentration; Bottom = the minimum response; Top = the maximum response; log EC_{50} = the logarithm of X as the response midway between Top and Bottom; and HillSlope describes the steepness of the curve. The model calculates the best fit for the Top, Bottom, HillSlope, and EC_{50} parameters. See **Section 11.6.5** for more details.

Acceptance or rejection of a test is based on evaluation of reference standard and control results from each experiment conducted on a 96-well plate. Results for these controls are compared to historical results compiled in the historical database, as seen in **Section 14.0**.

6.1 Range Finder Testing

Agonist range finding for coded substances consists of a seven point, 1:10 serial dilution using duplicate wells per concentration. Concentrations for comprehensive testing are selected based on the response observed in range finder testing. If necessary, a second range finder test can be conducted to clarify the optimal concentration range to test (see **Section 12.0**).

6.2 Comprehensive Testing

Comprehensive agonist testing for coded substances consists of 11 point, serial dilutions, with each concentration tested in triplicate wells of the 96-well plate. Three separate experiments are conducted for comprehensive testing on three separate days, except during Phases III and IV of the validation effort, in which comprehensive testing experiments are conducted once (see **Section 13.0**).

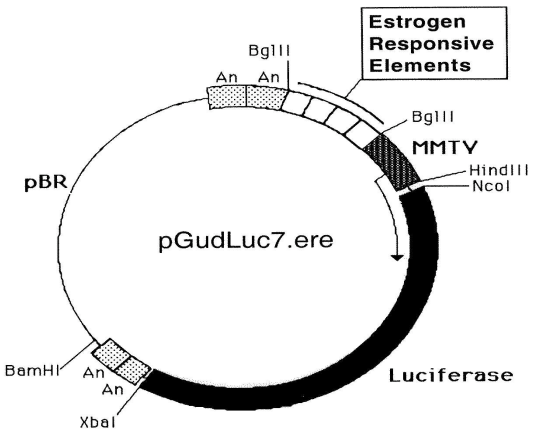
7.0 MATERIALS FOR LUMI-CELL® ER AGONIST TESTING

This section provides the materials needed to conduct LUMI-CELL® ER testing, with associated brand names/vendors[2] in brackets.

7.1 BG1Luc4E2 Cells:

Human ovarian cancer cell line stably transfected with a plasmid containing an estrogen response element pGudLuc7.0 (**Figure 7-1**) [XDS].

Figure 7-1 pGudLuc7.ERE Plasmid.



7.2 Technical Equipment:

All technical equipment may be obtained from Fisher Scientific International, Inc. (Liberty Lane Hampton, NH, USA 03842). Equivalent technical equipment from another commercial source can be used.

- Analytical balance (Cat. No. 01-910-320)
- Berthold Orion 1 Microplate Luminometer [Berthold CatNo.: Orion 1 MPL3] or equivalent and dedicated computer
- Biological safety hood, class II, and stand (Cat. No. 16-108-99)

[2]Brand names and vendors should not be considered an endorsement by the U.S. Government or any member of the U.S. Government; such information is provided as examples.

- Centrifuge (low speed, tabletop with swinging bucket rotor) (Cat. No. 04-978-50 centrifuge, and 05-103B rotor)
- Combustion test kit (CO_2 monitoring) (Cat. No. 10-884-1)
- Drummond diaphragm pipetter (Cat. No. 13-681-15)
- Freezers, –20°C (Cat. No. 13-986-150), and –70°C (Cat. No. 13-990-86)
- Hand tally counter (Cat. No. 07905-6)
- Hemocytometer, cell counter (Cat. No. 02-671-5)
- Light microscope, inverted (Cat. No. 12-561-INV)
- Light microscope, upright (Cat. No. 12-561-3M)
- Liquid nitrogen flask (Cat. No. 11-675-92)
- Micropipetter, repeating (Cat. No. 21-380-9)
- Pipetters, air displacement, single channel (0.5 –10µl (Cat. No. 21-377-191), 2 – 20 µl (Cat. No. 21-377-287), 20 – 200 µl (Cat. No. 21-377-298), 200 - 1000 µl (Cat. No. 21-377-195))
- Refrigerator/freezer (Cat. No. 13-986-106A)
- Shaker for 96-well plates (Cat. No. 14-271-9)
- Sodium hydroxide (Cat. No. 5318-500)
- Sonicating water bath (Cat. No. 15-335-30)
- Tissue culture incubator with CO_2 and temperature control (Cat. No. 11-689-4)
- Vacuum pump with liquid trap (side arm Erlenmeyer) (Cat. No. 01-092-29)
- Vortex mixer (Cat. No. 12-814)

Equipment should be maintained and calibrated as per GLP guidelines and individual laboratory SOPs.

7.3 Reference Standard, Controls, and Tissue Culture Supplies

All tissue culture reagents must be labeled to indicate source, identity, storage conditions and expiration dates. Tissue culture solutions must be labeled to indicate concentration, stability (where known), and preparation and expiration dates.

Equivalent tissue culture media and sera from another commercial source can be used, but must first be tested as described in **Section 15.0** to determine suitability for use in this test method.

The following are the necessary tissue culture reagents and possible commercial sources (in brackets) based on their use in the pre-validation studies:

- BackSeal-96/384, white adhesive bottom seal for 96-well and 384-well microplate [Perkin-Elmer, Cat. No. 6005199]
- 17 β-estradiol (CAS RN: 50-28-2) [Sigma-Aldrich, Cat. No. E8875]
- Cryovial, 2 mL (Corning Costar) [Fisher Scientific Cat. No. 03-374-21]
- Culture tube 13 x 100mm (case) [Thomas Scientific Cat. No.: 10009186R38][3]
- Culture tube, 50 mL conical (Corning Costar) [Fisher Scientific Cat. No. 05-526C]
- DMSO, U.S.P. analytical grade. [Sigma-Aldrich, Cat. No. 34869-100ML]
- Dulbecco's Modification of Eagle's Medium (DMEM), containing 4.5 g/L glucose, with sodium pyruvate, without phenol red or L-glutamine [Mediatech/Cellgro, Cat. No. 17-205-CV]
- Fetal Bovine Serum [Mediatech/Cellgro Cat. No. MT 35-010-CV]
- Fetal Bovine Serum, charcoal/dextran treated, triple 0.1 μm sterile filtered [Hyclone, Cat. No. SH30068.03]
- Gentamycin Sulfate (G418), 50 mg/mL [Mediatech/Cellgro Cat. No. 30-234-CR]
- L-glutamine, 29.2 mg/mL [Cellgro, Cat. No. 25005-CI]

[3] If glass tubes can not be obtained from Thomas Scientific, the preference is for flint glass, then lime glass, then borosilicate glass.

- Luciferase Assay System (10-Pack) [Promega Cat. No. E1501]
- Lysis Solution 5X [Promega, Cat. No. E1531]
- Methoxychlor (CAS RN: 72-43-5) [Sigma-Aldrich, Cat. No. 49054]
- Penicillin/streptomycin solution, 5000 I.U. penicillin, 5000 µg/mL streptomycin [Cellgro, Cat. No. 30-001-CI].
- Phosphate buffered saline (PBS, 1X) without calcium and magnesium [Cellgro, Cat. No. 21-040-CV]
- Pipettes, serological: 2.0 mL [Sigma-Aldrich, Cat. No. P1736], 5.0 mL [Sigma-Aldrich, Cat. No. P1986], 25 mL [Sigma-Aldrich, Cat. No. P2486]
- RPMI 1640 medium, containing L-glutamine [Mediatech, Cat. No. 10-040-CV]
- Tissue culture flasks (Corning-Costar): 25 cm^2 (T25) [Fisher Cat. No. 10-126-28]; 75 cm^2 (T75) [Fisher Cat. No. 10-126-37]; and 150 cm^2 (T150) [Fisher Cat. No. 10-126-34]
- Tissue culture plates (Corning-Costar): 96-well [Thomas Scientific Cat. No. 6916A05]
- Trypsin (10X), 2.5% in Hank's balanced salt solution (HBSS), without calcium and magnesium, without phenol red [Cellgro, Cat. No. 25-054-CI].

All reagent lot numbers and expiration dates must be recorded in the study notebook.

8.0 PREPARATION OF TISSUE CULTURE MEDIA AND SOLUTIONS

All tissue culture media and media supplements must be quality tested before use in experiment (see Section 15.0).

8.1 RPMI 1640 Growth Medium (RPMI)

RPMI 1640 is supplemented with 0.9% Pen-Strep and 8.0% FBS to make RPMI growth medium (RPMI).

Procedure for one 549 mL bottle:

1. Remove FBS from -70°C freezer, and Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.
2. Add 44 mL of FBS and 5 mL Pen-Strep to the bottle of RPMI 1640.
3. Label RPMI bottle as indicated in **Section 7.3**

Store at 2-8°C for no longer than six months or until the shortest expiration date of any media component.

8.2 Estrogen-Free DMEM Medium

DMEM is supplemented to contain 4.5% charcoal/dextran treated FBS, 1.9% L-glutamine, 0.9% Pen-Strep.

Procedure for one 539 mL bottle:

1. Remove charcoal/dextran treated FBS from -70°C freezer, and L-glutamine and Pen-Strep from -20°C freezer and allow to equilibrate to room temperature.
2. Add 24 mL of charcoal/dextran treated FBS, 10 mL L-glutamine, and 5 mL Pen-Strep to one 500 mL bottle of DMEM.
3. Label estrogen-free DMEM bottle as indicated in **Section 7.3**

Store at 2-8°C for no longer than six months or until the shortest expiration date of any media component..

8.3 1X Trypsin Solution

1X Trypsin solution is prepared by dilution from a 10X premixed stock solution. The 10X stock solution should be stored in 10 mL aliquots in a -20°C freezer.

Procedure for making 100 mL of 1X trypsin:

1. Remove a 10 mL aliquot of 10X trypsin from -20°C freezer and allow to equilibrate to room temperature.
2. Aliquot 1 mL Trypsin (10X) along with 9 mL of 1X PBS into ten 15 mL sterile centrifuge tubes.
3. Label 1X trypsin aliquots as indicated in **Section 7.3**

1X Trypsin should be stored at -20°C.

8.4 1X Lysis Solution

Lysis solution is prepared by dilution from a 5X premixed stock solution. Both the 5X and 1X solutions can be repeatedly freeze-thawed.

The procedure for making 10 mL of 1X lysis solution:

1. Thaw the 5X Promega Lysis solution and allow it to reach room temperature.
2. Remove 2 mL of 5X solution and place it in a 15 mL conical centrifuge tube.
3. Add 8 mL of distilled, de-ionized water to the conical tube.
4. Cap and shake gently until solutions are mixed.

Store at -20°C for no longer than 1 year from receipt.

8.5 Reconstituted Luciferase Reagent

Luciferase reagent consists of two components, luciferase buffer and lyophilized luciferase substrate.

For long term storage, unopened containers of the luciferase buffer and lyophilized luciferase substrate can be stored at -70°C for up to one year.

To reconstitute luciferase reagent:

1. Remove luciferase buffer and luciferase substrate from -70°C freezer and allow them to equilibrate to room temperature.
2. Add 10 mL of luciferase buffer solution to luciferase substrate container and swirl or vortex gently to mix; the Luciferase substrate should readily go into solution.
3. After solutions are mixed, aliquot to a 15mL centrifuge tube.
4. Store complete solution at –20°C.

Reconstituted luciferase reagent is stable for up to 1 month at – 20°C.

9.0 OVERVIEW OF PROPAGATION AND EXPERIMENTAL PLATING OF BG1Luc4E2 CELLS

The BG1Luc4E2 (BG-1) cells are stored in liquid nitrogen in 2 mL cryovials. BG-1 cells are grown as a monolayer in tissue culture flasks in a dedicated tissue culture incubator at 37ºC ± 1ºC, 90% ± 5% humidity, and 5.0% ± 1% CO_2/air. The cells should be examined, on a daily basis during working days, under an inverted phase contrast microscope and any changes in morphology and/or adhesive properties must be noted in the study notebook.

Two T150 flasks containing cells at 80 to 90% confluence will usually yield a sufficient number of cells to fill three 96-well plates for use in experiments.

9.1 Procedures for Thawing Cells and Establishing Tissue Cultures

Warm all of the tissue culture media and solutions to room temperature by placing them under the tissue culture hood several hours before use.

All tissue culture media, media supplements, and tissue culture plasticware must be quality tested before use in experiments (**Section 15.0**).

9.1.1 Thawing Cells

1. Remove a cryovial of frozen BG-1 cells from the liquid nitrogen flask.
2. Facilitate rapid thawing by loosening the top slightly (do not remove top) to release trapped gasses and retightening it. Roll vial between palms.
3. Use a micropipette to transfer cells to a 50 mL conical centrifuge tube.
4. Rinse cryovial twice with 1X PBS and add PBS rinse material to the conical tube.
5. Add 20 mL of RPMI to the conical tube.
6. Centrifuge at 1000 x *g* for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
7. Aspirate media from pellet and re-suspend it in 5 mL RPMI, drawing the pellet repeatedly through a 1.0 mL serological pipette to break up any clumps of cells.
8. Transfer cells to a T25 flask, place them in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

9.1.2 Establishing Tissue Cultures

Once cells have reached 80 to 90% confluence, transfer the cells to a T75 flask by performing, for example, the following steps:

1. Remove the T25 flask from the incubator.
2. Aspirate the RPMI, then add 5 mL 1X PBS, making sure that the cells are coated with PBS.
3. Aspirate 1X PBS, then add 1 to 2 mL 1X trypsin to the T25 flask, gently swirling the flask to coat all cells with the trypsin.
4. Place the flask in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
7. After cells have detached, add 5 mL PBS, and transfer the suspended cells to a 50 mL centrifuge tube. Wash the flask one additional time with 5 mL PBS.
8. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular digestion by residual trypsin.
9. Pellet the cells by centrifugation, as described in **Section 9.1.1**, and re-suspend the cells in 10 mL RPMI medium.
10. Draw the pellet repeatedly through a 25 mL serological pipette to break up clumps of cells
11. Transfer cells to a T75 flask, then place the flask in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

When cells have reached 80% to 90% confluency, transfer them into a T150 flask by performing, for example, the following steps:

12. Remove the T75 flask from the incubator, aspirate the old media and add 5 mL 1X PBS.
13. Aspirate 1X PBS, add 2 mL of 1X trypsin to the flask, and place it in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
14. Repeat steps **5** through **11** in **Section 9.1.2,** re-suspending the pellet in 20 mL of RPMI.
15. Transfer cells to a T150 flask and place it in the incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
16. Remove the T150 flask from the incubator.
17. Aspirate the RPMI and add 5 mL 1X PBS.
18. Aspirate 1X PBS and add 3 mL 1X trypsin to the T150 flask, making sure that the cells are coated with the trypsin.
19. Incubate cells in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
20. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
21. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
22. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask, swirl around the flask, and then transfer the PBS to the 50 mL conical tube.
23. Immediately add 20 mL RPMI to the conical tube to inhibit further cellular digestion by residual trypsin.
24. Centrifuge at 1000 x *g* for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.

25. Aspirate the media from the pellet and re-suspend it in 40 mL RPMI, drawing the pellet repeatedly through a 25 mL serological pipette to break up any clumps of cells.

26. Transfer 20 mL of cell suspension to each of two T150 flasks, place them in an incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

9.2 Ongoing Tissue Culture Maintenance, Conditioning in Estrogen-free Medium, and Plating Cells for Experimentation

The following procedure is used to condition the BG1Luc4E2 cells to an estrogen-free environment prior to plating the cells in 96-well plates for analysis of estrogen dependent induction of luciferase activity.

To start the tissue culture maintenance and estrogen-free conditioning, split the two T150 culture flasks into four T150 flasks. Two of these flasks will be used for continuing tissue culture and will use the RPMI media mentioned above. The other two flasks will be cultured in estrogen-free DMEM for experimental use. Extra care must be taken to avoid contaminating the estrogen-free cells with RPMI.

1. Remove both T150 flasks from the incubator.
2. Aspirate the medium and rinse the cells with 5 mL 1X PBS.
3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat all cells with the trypsin.
4. Incubate cells in the incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or heel of the hand.
6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for an additional 2 minutes, then hit the flask again.
7. After cells have detached, add 5 mL 1X PBS to the first T150 flask and transfer the suspended cells to the second T150 flask.

8. Transfer the contents of both flasks to a 50 mL conical tube. Repeat step 7 with an additional 5 mL 1X PBS and transfer to the 50 mL conical tube.
9. Immediately add 20 mL estrogen-free DMEM to the 50 mL conical tube to inhibit further cellular digestion by residual trypsin.
10. Centrifuge at 1000 x *g* for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
11. Aspirate media from pellet and re-suspend it in 4 mL estrogen-free DMEM, drawing the pellet repeatedly through a 1 mL serological pipette to break up clumps of cells.

At this point, cells are ready to be divided into the ongoing tissue culture and estrogen-free conditioning groups.

9.2.1 Ongoing Tissue Culture Maintenance

1. Add 20 mL RPMI to two T150 flasks.
2. Add 220 µl G418 to the RPMI in the T150 flasks
3. Add 1 mL of cell suspension from **9.2** step **11** to each flask.
4. Place T150 flasks in tissue culture incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).
5. Tissue culture medium may need to be changed 24 hours after addition of G418 to remove cells that have died because they do not express reporter plasmid.
6. G418 does not need to be added to the flasks a second time.
7. Repeat **Section 9.2 steps 1-11** for ongoing tissue culture maintenance.

9.2.2 Conditioning in Estrogen-free Medium

1. Add 20 mL estrogen-free DMEM to two T150 flasks.
2. Add 150 µL G418 to the estrogen-free DMEM in the T150 flasks.
3. Add 1 mL of cell suspension from **Section 9.2 step 11** to each flask.

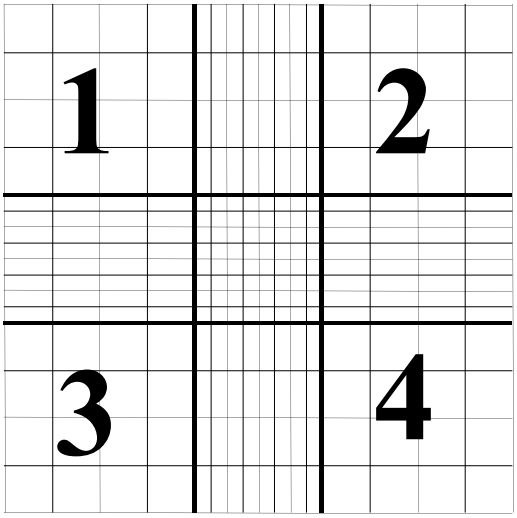
4. Tissue culture medium may need to be changed 24 hours after addition of G418 to remove cells that have died because they do not express reporter plasmid.
5. G418 does not need to be added to the flasks a second time.
6. Place the T150 flasks in the incubator (see conditions in **Section 9.0**) and grow to 80% to 90% confluence (approximately 48 to 72 hrs).

9.2.3 Plating Cells Grown in Estrogen-free DMEM for Experimentation

1. Remove the T150 flasks that have been conditioned in estrogen-free DMEM for 48 to 72 hours from the incubator.
2. Aspirate the medium, then rinse the cells with 5 mL 1X PBS.
3. Aspirate 1X PBS, then add 3 mL 1X trypsin to the flasks, gently swirling the flask to coat all cells with the trypsin.
4. Place the flasks in an incubator (see conditions in **Section 9.0**) for 5 to 10 min.
5. Detach cells by hitting the side of the flask sharply against the palm or the heel of the hand.
6. Confirm cell detachment by examination under an inverted microscope. If cells have not detached, return the flask to the incubator for 2 additional minutes, then hit the flask again.
7. After cells have detached, add 5 mL 1X PBS and transfer the suspended cells from the T150 flask to a 50 mL conical tube. Add an additional 5 mL PBS to the flask, gently swirl around the flask, and then transfer to the 50 mL conical tube.
8. Immediately add 20 mL estrogen-free DMEM to each conical tube to inhibit further cellular digestion by residual trypsin.
9. Centrifuge at 1000 x *g* for eight min. If a pellet of cells has not formed, centrifuge for an additional 5 minutes.
10. Aspirate the media from the pellet and re-suspend it in 20 mL DMEM, drawing the pellet repeatedly through a 25 mL serological pipette to break up any clumps of cells.

11. Pipette 15 µL of the cell suspension into the "v" shaped slot on the hemocytometer. Ensure that the solution covers the entire surface area of the hemocytometer grid, and allow cells to settle before counting.
12. Using 100x magnification, view the counting grid.
13. The counting grid on the hemocytometer consists of nine sections, four of which are counted (upper left, upper right, lower left, and lower right, see **Figure 9-1).** Each section counted consists of four by four grids. Starting at the top left and moving clockwise, count all cells in each of the four by four grids. Some cells will be touching the outside borders of the square, but only count those that touch the top and right borders of the square. This value is then used in the calculation below to get to the desired concentration of 200,000 cells/mL.

Figure 9-1 Hemocytometer Counting Grid.



The volume of each square is 10^{-4} mL, therefore:

Cells/mL=(average number per grid) x 10^{-4} mL x 1/(starting dilution).

<u>Starting dilution: 20 mL (for T150 flasks)</u>

Harvested cells for a T150 flask are suspended in 20 mL of estrogen-free DMEM and sampled for determination of concentration of cells/mL.

Example Calculation:

- Grids 1, 2, 3, and 4 are counted and provide the following data:
    - 50, 51, 49, and 50: average number of cells per grid is equal to 50.

Cells/mL = 50 cells per grid ÷ 10^{-4} mL volume of grid = 50 X 10^{4} cells/mL (or 500,000 cells/mL)

Total # of Cells Harvested = 500,000 cells/mL x 20 mL

Desired Concentration (or Concentration $_{Final}$)= 200,000 cells/mL

Formula: (Concentration $_{Final}$ x Volume $_{Final}$ = Concentration $_{Initial}$ x Volume $_{Initial}$)

Concentration $_{Final}$ = 200,000 cells/mL

Concentration $_{Initial}$ = 500,000 cells/mL

Volume $_{Initial}$ = 20 mL

Volume $_{Final}$ – to be solved for.

Therefore: 200,000 cells/mL x Volume $_{Final}$ = 500,000 cells/mL x 20 mL

Solving for Volume $_{Final}$ we find = 50 mL

Therefore, add 30 mL of estrogen-free DMEM to the cell suspension for a total volume of 50 mL, which will yield the desired concentration of 200,000 cells/mL for plating.

14. This dilution scheme will give a concentration of 200,000 cells/mL. 200 µL of this cell suspension is used for each well of a 96-well plate (i.e., 40,000 cells per well).

15. Remove a 96-well plate from its sterile packaging. Use a repeater pipetter to pipette 200 µL of cell suspension into each well for to be used for the testing of coded substances, reference standard and controls (**note**: add 200 µL of estrogen-free DMEM only to any wells not being used for testing).

16. Incubate plate(s) in an incubator (see conditions in **Section 9.0**) for a minimum of 24 hours, but no longer than 48 hours before dosing.

Two T150 flasks containing cells at 80% to 90% confluence will typically yield sufficient cells to fill four 96-well plates.

10.0 PREPARATION OF TEST SUBSTANCES

The solvent used for dissolution of test substances is 100% DMSO. All test substances should be allowed to equilibrate to room temperature before being dissolved and diluted. Test substance solutions (except for reference standards and controls) should not be prepared in bulk for use in subsequent tests. Test substances are to be used within 24 hours of preparation. Solutions should not have noticeable precipitate or cloudiness.

All information on weighing, solubility testing, and calculation of final concentrations for test substances, reference standards and controls is to be recorded in the study notebook.

10.1 Determination of Test Substance Solubility

1. Prepare a 100 mg/mL solution of the test substance in 100% DMSO in a 4 mL conical tube.
2. Vortex to mix.
3. If the test substance does not dissolve at 100 mg/mL, prepare a 10 mg/mL solution and vortex as above.
4. If the test substance does not dissolve at 10 mg/mL solution, prepare a 1 mg/mL solution in a 4 mL conical tube and vortex as above.
5. If the test substance does not dissolve at 1 mg/mL, prepare a 0.1 mg/mL solution in a 4 mL conical tube and vortex as above.
6. Continue testing, using 1/10 less substance in each subsequent attempt until test substance is solubilized in DMSO.

Once the test substance has fully dissolved in 100% DMSO, the test substance is ready to be used for LUMI-CELL® ER testing.

The Testing Facility shall forward the results from the solubility tests assay to the SMT through the designated contacts in electronic format and hard copy upon completion of testing.

10.2 Preparation of Reference Standards, Control and Test Substances

All "dosing solutions" of test substance concentrations are to be expressed as μg/mL in the study notebook and in all laboratory reports.

All information on preparation of test substances, reference standards and controls is to be recorded in the study notebook.

10.2.1 Preparation of Reference Standard and Positive Control Stock Solutions

Stock solutions of E2 and methoxychlor are prepared in 100% DMSO and stored at room temperature for up to three years or until the expiration date listed in the certificate of analysis for that substance.

10.2.1.1 *E2 Stock Solution*

The final concentration of the E2 stock solution is 1.0×10^{-2} μg/mL. Prepare the E2 stock as shown in **Table 10-1**.

Table 10-1 Preparation of E2 Stock Solution

Step #	Action	DMSO	E2 Concentration
1	Make a 10 mg/mL stock solution in 100% DMSO in a 4mL vial.	-	10 mg/mL
2	Transfer 10 μL E2 solution from Step #1 to a new 4 mL vial.	Add 990 μL of 100% DMSO. Vortex to mix.	100 μg/mL
3	Transfer 10 μL E2 solution from Step #2 to a new 4mL vial.	Add 990 μL of 100% DMSO. Vortex to mix.	1 μg/mL
4	Transfer 10 μL E2 solution from Step #3 to a 13 mm test tube to create the working solution.	Add 990 μL of 100% DMSO. Vortex to mix.	1.0×10^{-2} μg/mL

10.2.1.2 *Methoxychlor Stock Solution*

The final concentration of the methoxychlor stock solution is 313 μg/mL.

To prepare the methoxychlor stock solution, proceed as follows:

1. Make a 10 mg/mL stock solution of Methoxychlor in 100% DMSO in a 4 mL vial.
2. Remove 94 μL of the methoxychlor solution and place it in a new 4 mL vial.

3. Add 2.906 mL of 100% DMSO to the 4mL vial and gently vortex to mix.

10.2.2 Preparation of Reference Standard and DMSO Control for Range Finder Testing

Range finder testing is conducted on 96-well plates using four concentrations of E2 in duplicate as the reference standard. Four replicate wells are used for the DMSO control. All wells on the 96 well plate are used during range finder testing.

Store dosing solutions at room temperature. Use within 24 hours of preparation.

10.2.2.1 *Preparation of E2 Reference Standard for Range Finder Testing*

To make E2 dosing solutions:

1. label four 4 mL conical tubes with numbers 1 through 4 and place them in a tube rack
2. label four 13 mm glass test tubes with numbers 1 through 4, place them in a tube rack and add 600 µL of estrogen-free DMEM to each tube

Prepare dilutions to give final concentrations of the E2 as shown in **Table 10-2**.

Table 10-2 Preparation of E2 Reference Standard Dosing Solution for Range Finder Testing

Tube Number	100% DMSO	E2[1]	Estrogen-free DMEM[2]	Final Volume	E2 Concentration
1	6 µL	6 µl of 1.0 x 10^{-2} µg/mL working solution	600 µL	606 µL	5.00 x 10^{-5} µL
2	18 µL	6 µL of 1.0 x 10^{-2} µg/mL working solution	600 µL	606 µL	1.25 x 10^{-5} µL
3	18 µL	6 µL from conical tube #2	600 µL	606 µL	3.13 x 10^{-6} µL
4	18 µL	6 µL from conical tube #3	600 µL	606 µL	7.83 x 10^{-7} µL

[1]Add specified volume of 100% DMSO and 6 µl of the specified E2 solution to labeled 4 mL conical tubes, and vortex.
[2]Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing DMEM and vortex.

10.2.2.2 *Preparation of DMSO Control for Range Finder Testing*

1. Add 10 µL of 100% DMSO to a 13 mm glass tube.
2. Add 1000 µL of estrogen-free DMEM to tube and vortex.

10.2.3 Preparation of Test Substance Dosing Solutions for Range Finder Testing

Range finder experiments are used to determine the concentrations of test substance to be used during comprehensive testing. Agonist range finding for coded substances consists of seven point, 1:10 serial dilutions run in duplicate.

To make dosing solutions for coded substances:

1. label seven 4 mL conical tubes with numbers 1 through 7 and place them in a tube rack
2. label seven 13 mm glass test tubes with numbers 1 through 7, place them in a tube rack and add 600 µL of estrogen-free DMEM to each tube

Prepare dilutions as shown in **Table 10-3**.

Table 10-3 Preparation of Test Substance Dosing Solutions for Range Finder Testing

Tube Number	100% DMSO	Test Substance[1]	Transfer	Estrogen-free DMEM	Final Volume
1	-	6 µL of test substance solution from **Section 10.1** step **10**	6 µL	600 µL	606 µL

Tube Number	100% DMSO	Test Substance[1]	Transfer	Estrogen-free DMEM	Final Volume
2	90 µL	10 µL of test substance solution from **Section 10.1** step **10**	6 µL	600 µL	606 µL
3	90 µL	10 µL from conical tube #2	6 µL	600 µL	606 µL
4	90 µL	10 µL from conical tube #3	6 µL	600 µL	606 µL
5	90 µL	10 µL from conical tube #4	6 µL	600 µL	606 µL
6	90 µL	10 µL from conical tube #5	6 µL	600 µL	606 µL
7	90 µL	10 µL from conical tube #6	6 µL	600 µL	606 µL

[1]Add specified volume of 100% DMSO and test substance solution to labeled 4 mL conical tubes, and vortex.
[2]Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing DMEM and vortex.

Determination of whether a substance is positive in range finder testing and selection of starting concentrations for comprehensive testing will be discussed in **Section 12.0**.

10.2.4 Preparation of Reference Standard and Positive Control Dosing Solutions for Comprehensive Testing

Comprehensive testing is conducted on 96-well plates using 11 concentrations of E2 in duplicate as the reference standard. Four replicate wells for the DMSO control and three replicate wells for the methoxychlor control are included on each plate.

Store dosing solutions at room temperature. Use within 24 hours of preparation.

10.2.4.1 *Preparation of E2 Reference Standard for Comprehensive Testing*

To make E2 dosing solutions:

1. label 11 4 mL conical tubes with numbers 1 through 11 and place them in a tube rack
2. label 11 13 mm glass test tubes with numbers 1 through 11, place them in a tube rack and add 600 µL of DMEM to each tube

Prepare dilutions to give final concentrations of E2 as shown in **Table 10-4**.

Table 10-4 Preparation of E2 Reference Standard Dosing Solution for Comprehensive Testing

Tube Number	100% DMSO	E2[1]	Estrogen-free DMEM[2]	Final Volume	E2 Concentration
1	-	6 µl of 1.0 x 10^{-2} µg/mL working solution	600 µL	606 µL	1.00 x 10^{-4} µL
2	6 µL	6 µL of 1.0 x 10^{-2} µg/mL working solution	600 µL	606 µL	5.00 x 10^{-5} µL
3	6 µL	6 µL from conical tube #2	600 µL	606 µL	2.50 x 10^{-5} µL
4	6 µL	6 µL from conical tube #3	600 µL	606 µL	1.25 x 10^{-5} µL
5	6 µL	6 µL from conical tube #4	600 µL	606 µL	6.25 x 10^{-6} µL
6	6 µL	6 µL from conical tube #5	600 µL	606 µL	3.13 x 10^{-6} µL
7	6 µL	6 µL from conical tube #6	600 µL	606 µL	1.56 x 10^{-6} µL
8	6 µL	6 µL from conical tube #7	600 µL	606 µL	7.83 x 10^{-7} µL
9	6 µL	6 µL from conical tube #8	600 µL	606 µL	3.92 x 10^{-7} µL
10	6 µL	6 µL from conical tube #9	600 µL	606 µL	1.95 x 10^{-7} µL
11	6 µL	6 µL from conical tube #10	600 µL	606 µL	9.78 x 10^{-8} µL

[1]Add specified volume of 100% DMSO and 6 µl of the specified E2 solution to labeled 4 mL conical tubes, and vortex.
[2]Transfer 6 µL of DMSO/E2 solution from 4 mL conical tube to labeled 13 mL glass tubes containing DMEM and vortex.

10.2.4.2 *Preparation of Methoxychlor Control Dosing Solution for Comprehensive Testing*

1. Add 10 µL of the 313 µg/mL methoxychlor to a 13 mm glass tube.
2. Add 1000 µL of estrogen-free DMEM to the tube and vortex.

10.2.4.3 *Preparation of DMSO Control Dosing Solution for Comprehensive Testing*

1. Add 10 µL of 100% DMSO to four 13 mm tubes (solvent/negative controls).
2. Add 1000 µL of estrogen-free DMEM to the tube and vortex.

10.2.5 Preparation of Test Substance Dosing Solutions for Comprehensive Testing

Comprehensive testing experiments are used to determine whether a substance possesses ER agonist activity in the LUMI-CELL® ER test method. Agonist comprehensive testing for coded substances consists of either an 11 point 1:2 serial dilution or an 11 point 1:5 serial dilution,

depending on the results from range finder testing **(see Section 12.0)** with each concentration tested in triplicate wells of the 96-well plate.

10.2.5.1 *Preparation of Test Substance 1:2 Serial Dilutions for Comprehensive Testing*

Start the 11-point serial dilution according to criteria in **Section 12.0**.

To make test substance 1:2 serial dilutions for comprehensive testing:

1. label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a tube rack
2. label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a tube rack and add 800 µL of estrogen-free DMEM to each tube

Prepare dilution of test substance as shown in **Table 10-6**.

Table 10-5 Preparation of Test Substance 1:2 Serial Dilutions for Comprehensive Testing

Tube Number	100% DMSO	Test Substance[1]	Transfer	Estrogen-free DMEM	Final Volume
1	-	8 µL of highest concentration of test substance solution	8 µL	800 µL	808 µL
2	8 µL	8 µL of highest concentration of test substance solution	8 µL	800 µL	808 µL
3	8 µL	8 µL from conical tube #2	8 µL	800 µL	808 µL
4	8 µL	8 µL from conical tube #3	8 µL	800 µL	808 µL
5	8 µL	8 µL from conical tube #4	8 µL	800 µL	808 µL
6	8 µL	8 µL from conical tube #5	8 µL	800 µL	808 µL
7	8 µL	8 µL from conical tube #6	8 µL	800 µL	808 µL
8	8 µL	8 µL from conical tube #7	8 µL	800 µL	808 µL
9	8 µL	8 µL from conical tube #8	8 µL	800 µL	808 µL
10	8 µL	8 µL from conical tube #9	8 µL	800 µL	808 µL
11	8 µL	8 µL from conical tube #10	8 µL	800 µL	808 µL

[1]Add specified volume of 100% DMSO and test substance solution to labeled 4 mL conical tubes, and vortex.

10.2.5.2 *Preparation of Test Substance 1:5 Serial Dilutions for Comprehensive Testing*

Start the 11-point serial dilution according to criteria in **Section 12.0**.

To make test substance 1:5 serial dilutions for comprehensive testing:

3. label eleven 4 mL conical tubes with numbers 1 through 11 and place them in a tube rack
4. label eleven 13 mm glass test tubes with numbers 1 through 11, place them in a tube rack and add 800 µL of estrogen-free DMEM to each tube

Prepare dilution of test substance as shown in **Table 10-6**.

Table 10-6 Preparation of Test Substance 1:5 Serial Dilutions for Comprehensive Testing

Tube Number	100% DMSO	Test Substance[1]	Transfer	Estrogen-free DMEM	Final Volume
1	-	8 µL of highest concentration of test substance solution	8 µL	800 µL	808 µL
2	16 µL	4 µL of highest concentration of test substance solution	8 µL	800 µL	808 µL
3	16 µL	4 µL from conical tube #2	8 µL	800 µL	808 µL
4	16 µL	4 µL from conical tube #3	8 µL	800 µL	808 µL
5	16 µL	4 µL from conical tube #4	8 µL	800 µL	808 µL
6	16 µL	4 µL from conical tube #5	8 µL	800 µL	808 µL
7	16 µL	4 µL from conical tube #6	8 µL	800 µL	808 µL
8	16 µL	4 µL from conical tube #7	8 µL	800 µL	808 µL
9	16 µL	4 µL from conical tube #8	8 µL	800 µL	808 µL
10	16 µL	4 µL from conical tube #9	8 µL	800 µL	808 µL
11	16 µL	4 µL from conical tube #10	8 µL	800 µL	808 µL

[1]Add specified volume of 100% DMSO and test substance solution to labeled 4 mL conical tubes, and vortex.

11.0 GENERAL PROCEDURES FOR THE TESTING OF CODED SUBSTANCES

Range finder experiments are used to determine the concentrations of test substance to be used during comprehensive testing. Comprehensive testing experiments are used to determine whether a substance possesses ER agonist activity in the LUMI-CELL® ER assay.

General procedures for range finder and comprehensive are similar. For specific details (such as plate layout) of range finder testing see **Section 12.0**. For specific details of comprehensive testing, see **Section 13.0**.

11.1 Application of Reference Standard, Controls, and Test Substances

1. Remove seeded 96-well plates from the incubator, inspect them using an inverted microscope. Only use plates in which the cells in all wells giving a score of 1 according to **Table 11-1**.
2. Remove medium by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.
3. Add 200 µL of reference standard, control, or test substance to each well (see **Sections 12.0** and **13.0** for specific plate layouts).
4. Return plates to incubator and incubate (see **Section 9.0** for details) for 19 to 24 hours to allow maximal induction of luciferase activity in the cells.

11.1.1 Preparation of Excel® Data Analysis Template For Range Finder Testing

1. In Excel®, open a new "AgRFTemplate" and save it with the appropriate project name as indicated in the NICEATM Style Guide.
2. Fill out the table at the top of the "Raw Data" worksheet with information regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot. Meas. Time/Well (s), etc. (**note**: this information can be permanently added to the default template "AgRFTemplate" on a laboratory specific basis).
3. Add the following information regarding the assay to the "Compound Tracking" worksheet.
    - Plate # - Enter the experiment ID or plate number into cell E1
    - Cell Lot # - Enter the passage or lot number of the cells used for this experiment into cell B5
    - DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and Media in cells B6 and B7

- Test Substance Code – Enter the test substance codes into cells C13 to C18
- Name: Enter the experimenter name into cell G6
- Date: Enter the experiment date in the format day\month\year into cell G10
- Comments: - Enter any comments about the experiment in this box (e.g., plate contaminated)

4. Enter the following substance testing information to the "List" page:
   - Concentration – Type in the test substance concentration in µg/ml in descending order.
   - Also add any replicate-specific comments on this page (e.g, spilled tube, etc.), in the comments section
   - All of the remaining cells on the List tab should populate automatically.
   - The "Template", "Compound Mixing" and "Visual Inspection" tabs should automatically populate with the information entered into the Compound Tracking and List tabs.
5. Save the newly named project file.
6. Print out either the "List" or "Template" page for help with dosing the 96-well plate. Sign and date the print out and store in study notebook.

11.1.2 Preparation of Excel® Data Analysis Template for Comprehensive Testing

1. In Excel®, open a new "AgCTTemplate" and save it with the appropriate project name as indicated in the NICEATM Style Guide.
2. Fill out the table at the top of the "Raw Data" worksheet with information regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot. Meas. Time/Well (s), etc. (**note**: this information can be permanently added to the default template "AgCTTemplate" on a laboratory specific basis).

3. On the "Compound Tracking" tab, enter the following information:
   - Plate # - Enter the experiment ID or plate number into cell E1
   - Cell Lot # - Enter the passage or lot number of the cells used for this experiment into cell C5
   - DMSO and Media Lot #'s – Enter the lot numbers for the DMSO and Media in cells C6 and C7
   - Test Substance Code – Enter the test substance codes into cells C15 and C16. Enter the test substance dilution into cells E25 and E26.
   - Name: Enter the experimenter name into cell G6
   - Date: Enter the experiment date in the format day\month\year into cell G10
   - Comments: - Enter any comments about the experiment in this box (e.g., plate contaminated)
4. Enter substance testing concentrations to the "List" page. Also add any replicate-specific comments on this page (e.g, spilled tube, etc.).
5. Save the newly named project file.
6. Print out either the "List" or "Template" page for help with dosing the 96-well plate. Sign and date the print out and store in study notebook.

11.2 Visual Evaluation of Cell Viability

1. 19 to 24 hours after dosing the plate, remove the plate from the incubator and remove the media from the wells by inverting the plate onto blotter paper. Gently tap plate against the bench surface to remove residual liquid trapped in the wells.
2. Use a repeat pipetter to add 50 μL 1X PBS to all wells. Immediately remove PBS by inversion.
3. Using an inverted microscope, inspect all of the wells used in the 96-well plate and record the visual observations using the scores in **Table 11-1**.

Table 11-1 Visual Observation Scoring

Viability Score	Brief Description[1]
1	Normal Cell Morphology and Cell Density
2	Altered Cell Morphology and/or Small Gaps between Cells
3	Altered Cell Morphology and/or Large Gaps between Cells
4	Few (or no) Visible Cells
P	Wells containing precipitation are to be noted with "P"

[1]Reference photomicrographs are provided in the LUMI-CELL® ER Validation Study "Visual Observation Cell Viability Manual."

11.3 Lysis of Cells for LUMI-CELL® ER

1. Apply the reflective white backing tape to the bottom of the 96-well plate (this will increase the effectiveness of the luminometer).
2. Add 30 µL 1X lysis reagent to the assay wells and place the 96-well plate on an orbital shaker for one minute.
3. Remove plate from shaker and measure luminescence (as described in **Section 11.4**).

11.4 Measurement of Luminescence

Luminescence is measured in the range of 300 to 650 nm, using an injecting luminometer and with software that controls the injection volume and measurement interval. Light emission from each well is expressed as RLU per well. The luminometer output is saved as raw data in an Excel® spread sheet. A hard copy of the luminometer raw data should be signed, dated and stored in the study notebook.

11.5 Data Analysis

LUMI-CELL® ER uses an Excel® spreadsheet to collect and adjust the RLU values obtained from the luminometer and a GraphPad Prism® template to analyze and graph data. The Excel® spreadsheet subtracts background luminescence (average DMSO solvent control RLU value) from test substance, reference standard and control RLU values. Plate induction is calculated using these corrected RLU values. Test substance, reference standard, and control RLU values are then adjusted relative to the highest E2 reference standard RLU value, which is set to 10,000. After adjustment, values are transferred to GraphPad Prism® for data analysis and graphing.

11.5.1 Collection and Adjustment of Luminometer Data for Range Finder Testing

The following steps describe the procedures required to populate the Excel® spreadsheet that has been configured to collect and adjust the RLU values obtained from the luminometer.

1. Open the raw data file and the corresponding experimental Excel® spreadsheet from **Section 11.1.1**.
2. Copy the raw data using the Excel® copy function, then paste the copied data into cell B19 of the "RAW DATA" tab in the experimental Excel® spreadsheet using the **Paste Special – Values** command. This position corresponds to position A1 in the table labeled Table 1 in this tab.
3. Examine the DMSO data in Table 1 of the Excel® spreadsheet to determine whether there are any potential outliers. See **Section 11.6.2** for further explanation of outlier determinations.
4. If an outlier is identified, perform the following steps to remove the outlier from calculations:
    - correct the equation used to calculate DMSO background in Table 1[e.g., if outlier is located in cell F26, adjust the calculation in cell H40 to read =AVERAGE(G26:I26)]
    - then correct the equation used to calculate the average DMSO value in Table 2 [e.g., following the above example, adjust cell M42 to read =AVERAGE(G26:I26)]
    - then correct the equation used to calculate the standard deviation of the DMSO value in Table 2 [e.g., following the above example, adjust cell M43 to read =STDEV(G36:I36)]
5. Excel® will automatically subtract the background (the average DMSO control value) from all of the RLU values in Table 1 and populate Table 2 with these adjusted values.
6. To calculate plate induction, identify the cell containing the E2a replicate in Table 1, plate row H that has the highest RLU value (i.e., cell B26, C26, D26, or E26).

7. Click into cell D14 and enter the cell number from the previous step into the numerator.
8. Identify the cell containing the E2b replicate in Table 1, plate row H that has the highest RLU value (i.e., cell J26, K26, L26, or M26).
9. Click into cell E14 and enter the cell number from the previous step into the numerator.
10. Click on the "ER Agonist Report" worksheet.
11. The data for the E2 reference standard, methoxychlor, and DMSO replicates populate the left portion (columns A – F) of the spreadsheet. The data is automatically placed in an Excel® graph.
12. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell D2 of "ER Agonist Report" tab and check the formula contained within that cell. The divisor should be the cell number of the cell containing the highest Mean E2 RLU value ((i.e., cell A16, A17, A18, or A19).
13. Open the "Visual Observation Scoring" worksheet. Enter the visual observation scores for each well on the 96-well plate. This data will be linked to the "ER Agonist Report" worksheet.
14. After the testing results have been evaluated and reviewed for quality control, enter the following information into the Compound Tracking worksheet:
    - Enter pass/fail results for plate reference standard and control parameters into the Plate Pass/Fail Table
    - Enter information from the testing of coded substances into the Testing Results Table
    - Reviewer Name – Enter the name of the person who Reviewed\QC'ed the data into cell A34
    - Date – Enter the date on which the data was reviewed into cell D34

11.5.2 Collection and Adjustment of Luminometer Data for Comprehensive Testing

The following steps describe the procedures required to populate the Excel® spreadsheet that has been configured to collect and adjust the RLU values obtained from the luminometer.

1. Open the raw data file and the corresponding experimental Excel® spreadsheet from **Section 11.1.2**.
2. Copy the raw data using the Excel® copy function, then paste the copied data into cell B16 of the "RAW DATA" worksheet in the experimental Excel® spreadsheet using the **Paste Special – Values** command. This position corresponds to position A1 in the table labeled Table 1 in this worksheet.
3. Fill out the table at the top of the "Raw Data" worksheet with information regarding the Microplate reader used, Reading Direction, No. of Intervals, Tot. Meas. Time/Well (s), etc. If desired, this information can be added to the Laboratory Template File.
4. Examine the DMSO data in Table 1 of the Excel® spreadsheet to determine whether there are any potential outliers. See **Section 11.6.2** for further explanation of outlier determinations.
5. If an outlier is identified, perform the following steps to remove the outlier from calculations:
    - correct the equation used to calculate DMSO background in Table 1[e.g., if outlier is located in cell M17, adjust the calculation in cell H37 to read =AVERAGE(M16,M18:M19)]
    - then correct the equation used to calculate the DMSO mean and SD values [e.g., following the above example, adjust cell M39 to read =AVERAGE(M28,M30:M31), and adjust cell M40 to read =STDEV(M28,M30:M31)]
6. Excel® will automatically subtract the background (the average DMSO control value) from all of the RLU values in Table 1 and populate Table 2 with these adjusted values.

7. To calculate plate induction, identify the cell in containing the E2 replicate in Table 1, plate row G that has the highest RLU value.
8. Click into cell D11 and enter the cell number from the previous step into the numerator.
9. Identify the cell containing the E2 replicate in plate row H that has the highest RLU value.
10. Click into cell E11 and enter the cell number from the previous step into the numerator.
11. Open the "ER Agonist Report" worksheet.
12. The data for the E2 reference standard, methoxychlor, and DMSO replicates populate the left portion (columns A – E) of the spreadsheet. The data is automatically placed in an Excel® graph.
13. To set the highest RLU value for the reference standard to 10,000 RLU, go to cell E2 of "ER Agonist Report" tab and check the formula contained within that cell. The divisor should be the cell number of the cell containing the highest Avg E2 RLU value (cells A16 through A26).
14. Open the "Visual Observation Scoring" worksheet. Enter the visual observation scores for each well on the 96-well plate. This data will be linked to the "ER Agonist Report" worksheet.
15. Copy the data from the "ER Agonist Report" worksheet into GraphPad Prism® for the calculation of EC_{50} values and to graph experimental results as indicated in the NICEATM Prism® Users Guide.
16. After the testing results have been evaluated and reviewed for quality control, enter the following information into the Compound Tracking worksheet:
    - Enter pass/fail results for plate reference standard and control parameters into the Plate Pass/Fail Table
    - Enter information from the testing of coded substances into the Testing Results Table

- Reviewer Name – Enter the name of the person who Reviewed\QC'ed the data into cell A32
- Date – Enter the date on which the data was reviewed into cell D32

11.5.3 Determination of Outliers

The Study Director will use good statistical judgment for determining "unusable" wells that will be excluded from the data analysis and will provide an explanation in the study notebook for any excluded data. This judgment for data acceptance will include Q-test analysis.

The formula for the Q test is:

$$\frac{Outlier - Nearest\ Neighbor}{Range\ (Highest - Lowest)}$$

where the outlier is the value proposed for exclusion, the nearest neighbor is the value closest to the outlier, and the range is the range of the three values (Q values for samples sizes from 3 to 10 are provided in **Table 11-2**). For example, if the value of this ratio is greater than 0.94 (the Q value for the 90% confidence interval for a sample size of three) or 0.76 (the Q value for the 90% confidence interval for a sample size of four), the outlier may be excluded from data analysis.

Table 11-2 Q Test Values

Number Of Observations	Q Value
2	**-**
3	**0.94**
4	**0.76**
5	**0.64**
6	**0.56**
7	**0.51**
8	**0.47**
9	**0.44**
10	**0.41**

For E2 reference standard replicates (sample size of two), any adjusted RLU value for a replicate at a given concentration of E2 is considered and outlier if its value is more than 20% above or below the adjusted RLU value for that concentration in the historical database.

11.5.4 Acceptance Criteria

11.5.4.1 *Range Finder Testing*

Acceptance or rejection of a test is based on evaluation of reference standard and control results from each experiment conducted on a 96-well plate. Results are compared to quality controls (QC) for these parameters derived from the historical database, which are summarized below.

- Induction: Plate induction, as measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control RLU value, must be greater than three-fold.
- DMSO control results: Solvent control RLU values must be within 2.5 times the standard deviation of the historical solvent control mean RLU value.

An experiment that fails either acceptance criterion will be discarded and repeated.

11.5.4.2 *Comprehensive testing*

Acceptance or rejection of a test is based on evaluation of reference standard and control results from each experiment conducted on a 96-well plate. Results are compared to quality controls (QC) for these parameters derived from the historical database, which are summarized below.

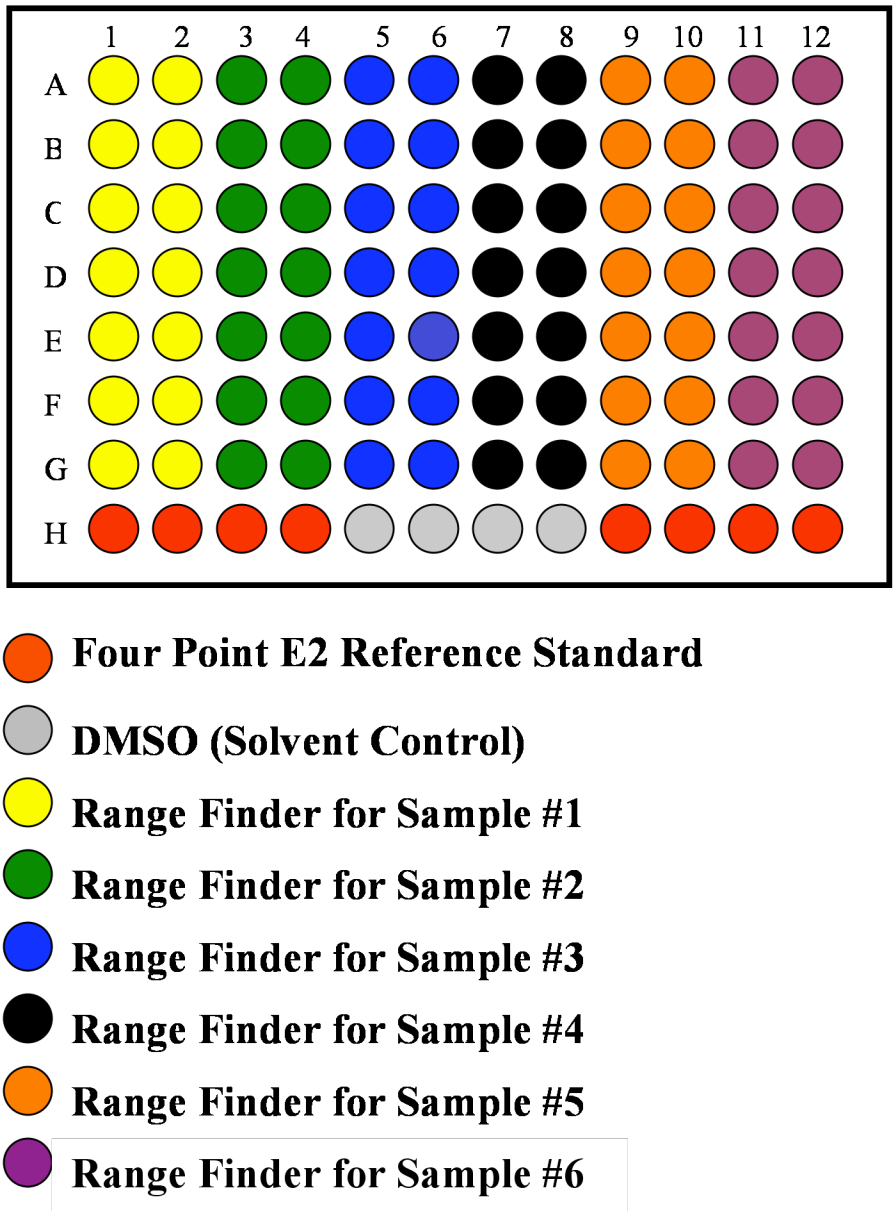
- Induction: Plate induction, as measured by dividing the averaged highest E2 reference standard RLU value by the averaged DMSO control RLU value, must be greater than three-fold.
- Reference standard results: The E2 reference standard concentration-response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration-response curve.
- DMSO control results: DMSO control RLU values must be within 2.5 times the standard deviation of the historical solvent control mean RLU value.
- Positive control results: Methoxychlor control RLU values must be above the line representing the DMSO mean plus three times the standard deviation from the DMSO mean.

An experiment that fails any single acceptance criterion will be discarded and repeated.

12.0 RANGE FINDER TESTING

Agonist range finding for coded substances consists of seven point, 1:10 serial dilutions, with each concentration tested in duplicate wells of the 96-well plate. **Figure 12-1** contains a template for the plate layout to be used in agonist range finder testing.

Figure 12-1 Agonist Range Finder Test Plate Layout



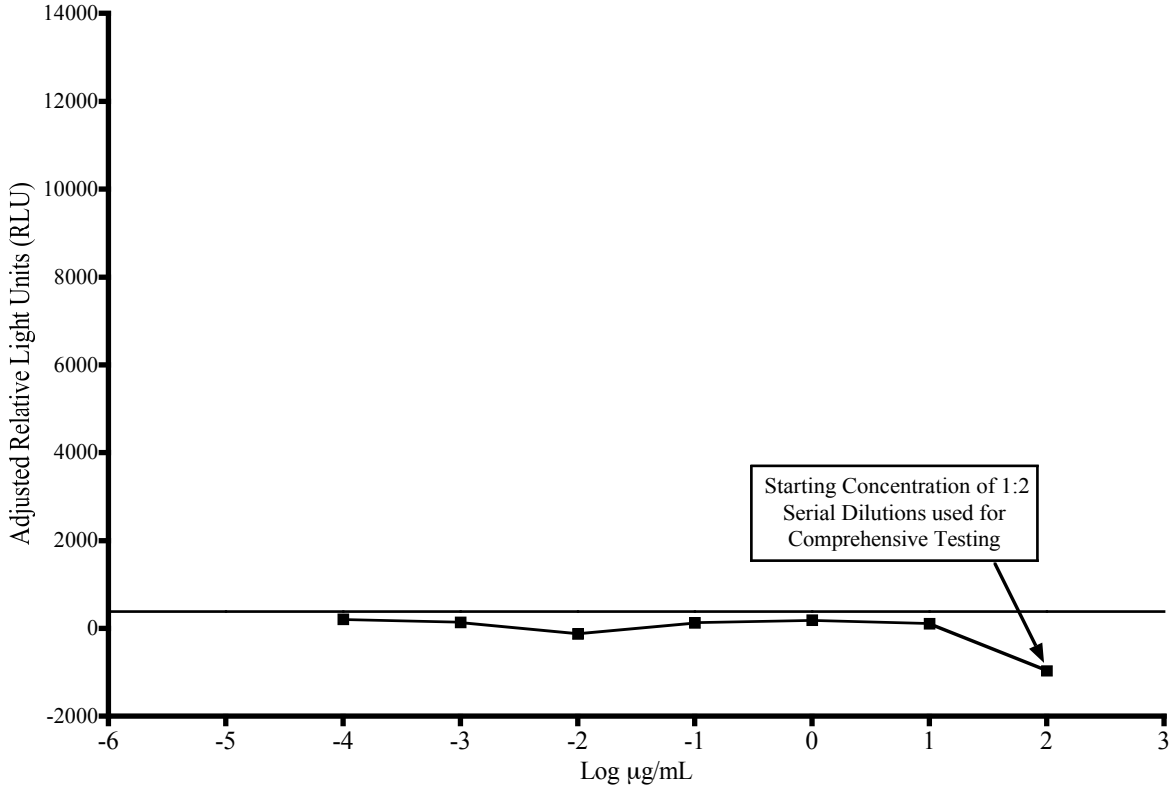
Evaluate whether range finder experiments have met the acceptance criteria (see **Section 11.5.4.1**).

To determine starting concentrations for comprehensive testing use the following criteria:

- If results in the range finder test suggest that the test substance is negative for agonist activity (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, see **Figure 12-2**), comprehensive testing will be conducted using an 11 point 1:2 serial dilution starting at the maximum soluble concentration.
- If results in the range finder test suggest that the test substance is negative for agonist activity (i.e., if there are no points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control), and the higher concentrations in the range finder are cytotoxic, comprehensive testing will be conducted using an 11 point 1:2 serial dilution with the lowest cytotoxic concentration as the starting concentration (see **Figure 12-3**).
- If results in the range finder test suggest that the test substance is positive for agonist activity (i.e., if there are points on the test substance concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control), the starting concentration to be used for the 11-point dilution scheme in comprehensive testing should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. The 11-point dilution scheme will be based on either 1:2 or 1:5 dilutions according to the following criteria:
  - An 11-point 1:2 serial dilution should be used if the resulting concentration range (note: an 11-point 1:2 serial dilution will cover a range of concentrations over approximately three orders of magnitude [three logs]) will encompass the full range of responses based on the concentration response curve generated in the range finder test (see **Figure 12-4**).
  - If the concentration range that would be generated with the 1:2 serial dilution will not encompass the full range of responses based on the concentration response curve in the range finder test (see **Figure 12-5** and **12-6**), an 11-point 1:5 serial dilution should be used instead.

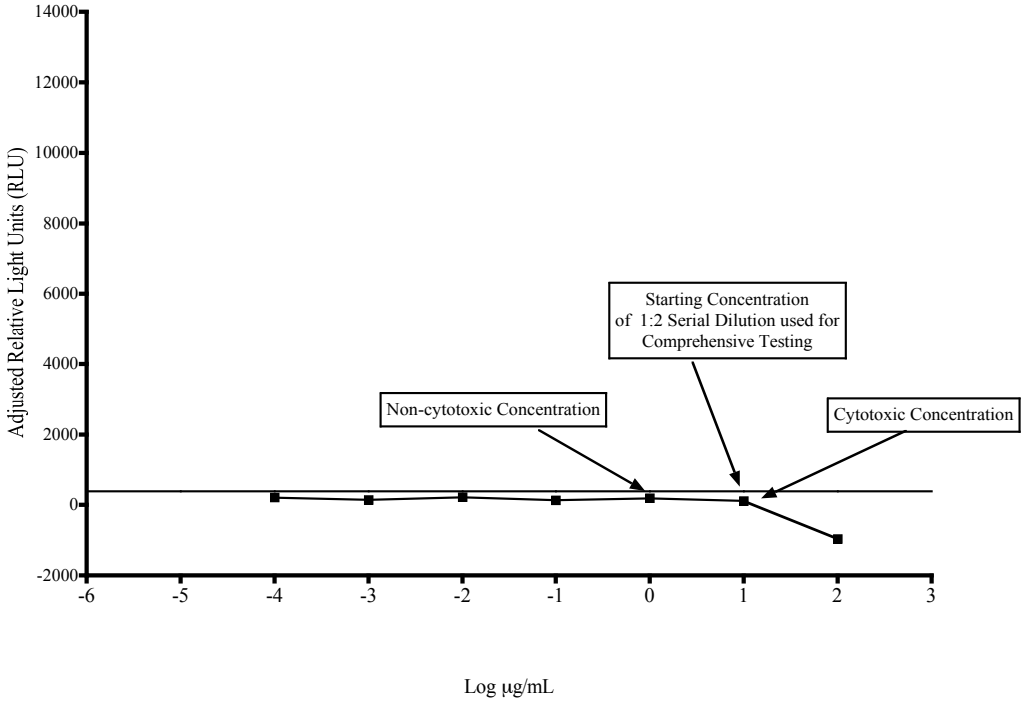
- If a substance exhibits a biphasic concentration response curve in the range finder test, both phases should also be resolved in comprehensive testing. In order to resolve both curves, the starting concentration should be based on the peak associated with the higher concentration and should be one log higher than the concentration giving the highest adjusted RLU value in the range finder. As an example, an 11-point 1:5 serial dilution should be used based on the range finder results presented in **Figure12-7**.

Figure 12-2 Agonist Range Finder (example 1)



The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

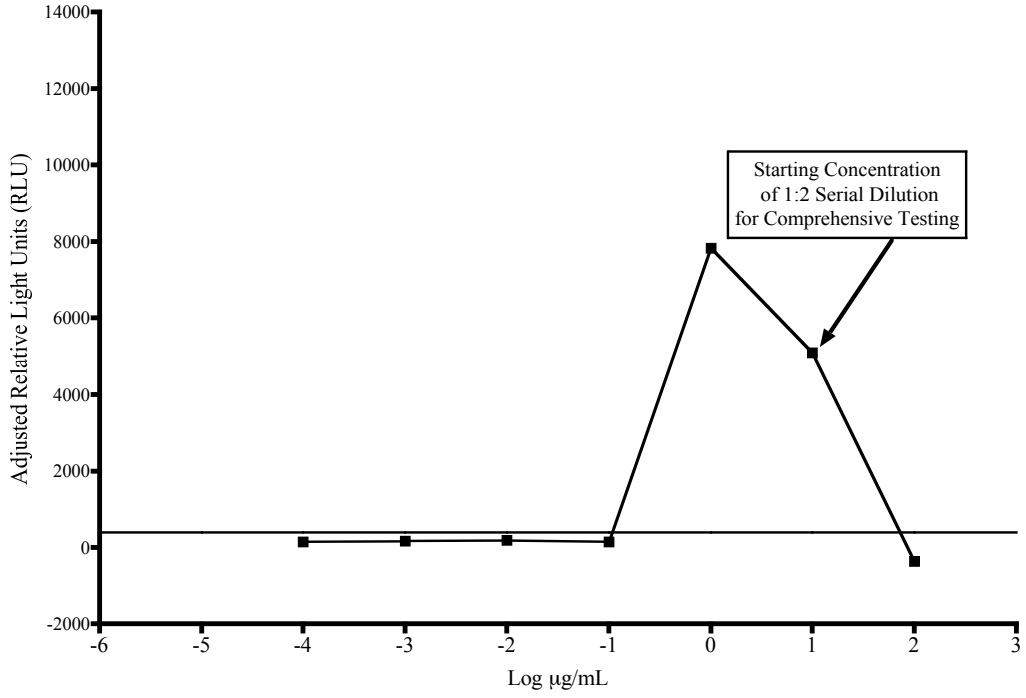
1129 **Figure 12-3 Agonist Range Finder (example 2)**



1130

1131 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

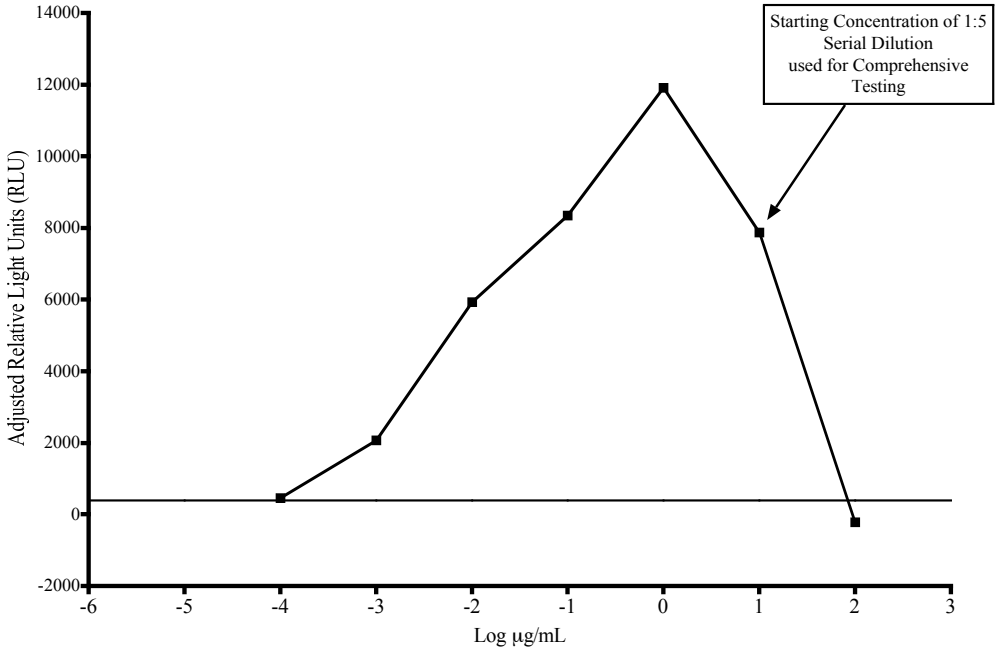
1132 **Figure 12-4 Agonist Range Finder (example 3)**



1133

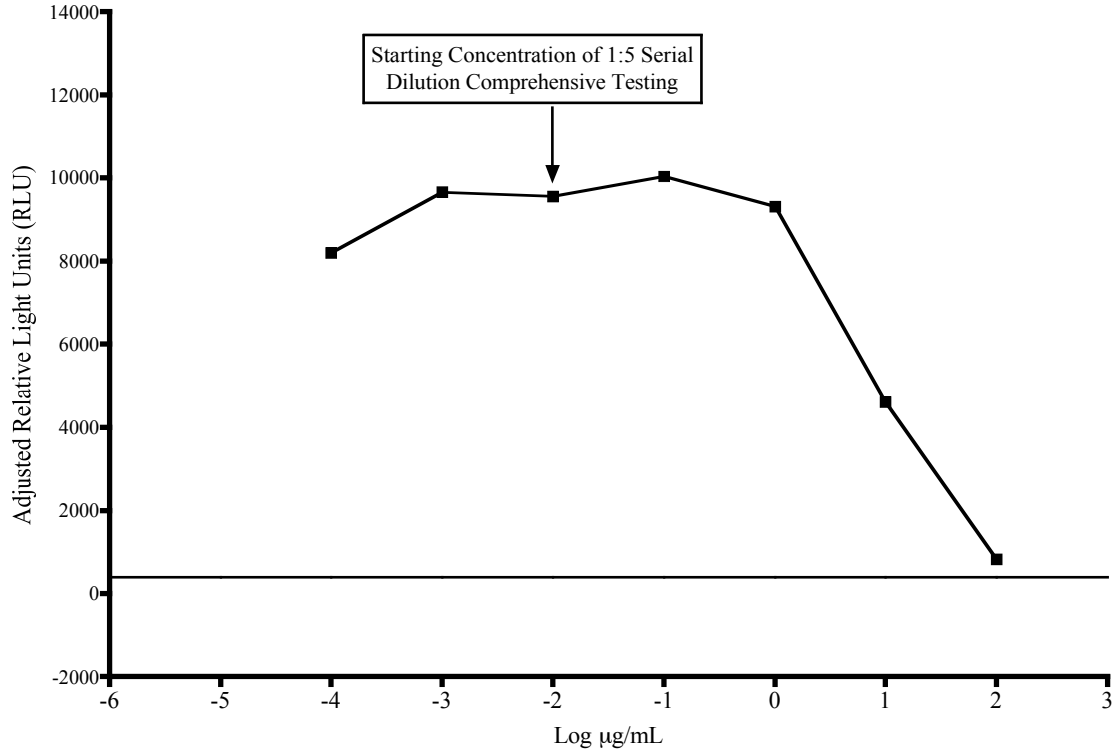
1134 The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

Figure 12-5 Agonist Range Finder (example 4)



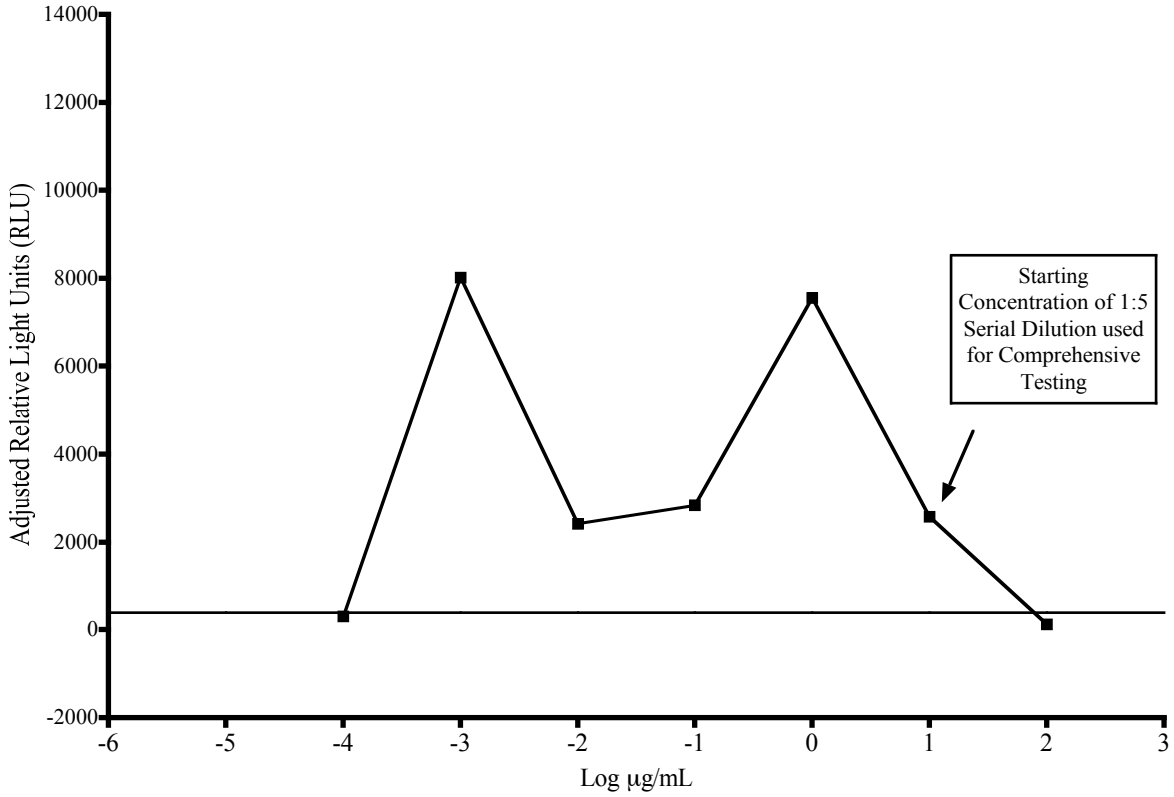
The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

Figure 12-6 Agonist Range Finder (example 5)



The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

Figure 12-7 Agonist Range Finder (example 6)

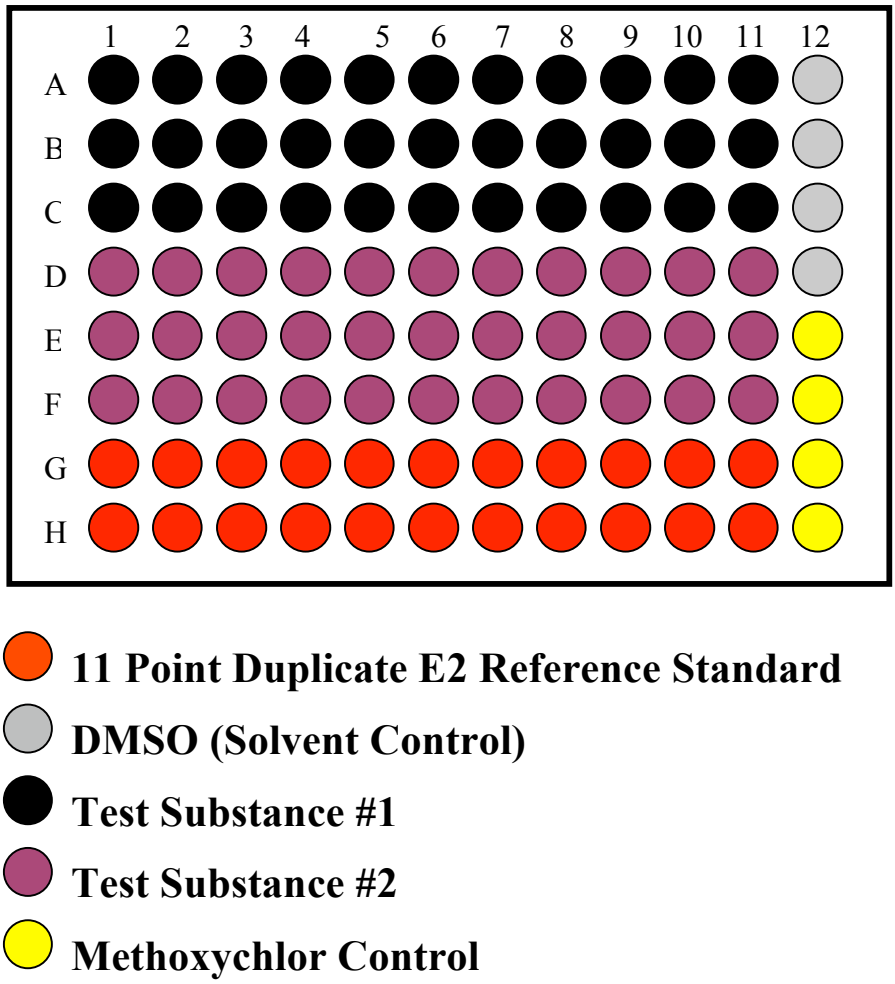


The solid horizontal line represents the mean plus three times the standard deviation of the DMSO control.

13.0 COMPREHENSIVE TESTING

Agonist comprehensive testing for coded substances consists of 11 point serial dilutions (either 1:2 or 1:5 serial dilutions based on the starting concentration for comprehensive testing criteria in **Section 12.0**) with each concentration tested in triplicate wells of the 96-well plate. **Figure 13-1** contains a template for the plate layout to be used in agonist comprehensive testing.

Figure 13-1 Agonist Comprehensive Test Plate Layout



Evaluate whether comprehensive experiments have met acceptance criteria (see **Section 11.6.4**) and graph the data as described in the NICEATM Prism® users guide.

- If the substance has been tested up to the limit dose or the maximum soluble dose, without causing a significant decrease in cell viability, and there are no points on the concentration curve that are greater than the mean plus three times the standard deviation of the DMSO control, the substance is considered negative for agonism
- If the substance has a positive response (See **Section 6.0**) at any concentration, the substance is considered positive for agonism.

14.0 COMPILATION OF THE HISTORICAL QUALITY CONTROL DATABASE

Historical databases are maintained in order to ensure that the assay is functioning properly. Historical databases are compiled using Excel® spreadsheets and are separate from the spreadsheets used to collect the data for individual test plates. Reference standard and control data are used to develop and maintain the historical database and are used as quality controls to determine acceptance of individual test plates.

The sources of the data needed to compile the historical database for the DMSO control are the experiment specific Excel® data collection and analysis spreadsheets used for LUMI-CELL® ER agonist and antagonist testing (see **Section 11.5.2** of the LUMI-CELL® ER agonist protocol and **Section 13.5.2** in the LUMI-CELL® ER antagonist protocol).

14.1 DMSO Control

Open the combined agonist and antagonist LUMI-CELL® ER historical database Excel® spreadsheet (LUMI_AgandAntQC.xls) and save under a new name using the Excel® "Save As" function, adding the laboratory designator to the file name (e.g., for Laboratory H, the new name would be HLUMI_AgandAntQC.xls). Enter the date and experiment name into worksheet columns A and B respectively. Enter the experimental mean DMSO control value (from cell H37 in the RAW DATA worksheet of the agonist and antagonist Excel® data collection and analysis spreadsheet) into worksheet column C. Acceptance or rejection of the plate DMSO control data for range finding and comprehensive testing is based on whether the mean plate DMSO RLU value falls within 2.5 times the standard deviation of the DMSO value in the historical database (columns G and H in the DMSO worksheet).

15.0 QUALITY TESTING OF MATERIALS

All information pertaining to the preparation and testing of media, media supplements, and other materials should be recorded in the Study Notebook.

15.1 Tissue Culture Media

Each lot of tissue culture medium must be tested in a single growth flask of cells before use in ongoing tissue culture or experimentation (**note:** each bottle within a given lot of Charcoal/Dextran treated FBS must be tested separately).

1. Every new lot of media (RPMI and DMEM) and media components (FBS, Charcoal/Dextran treated FBS, and L-glutamine) must first be tested on the LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.
2. Add 4 μL of DMSO (previously tested) into four separate 13 mm tubes.
3. Add 400 μL media (to be tested) to the same tubes.
4. Dose an experimental plate as in **Section 12.0,** treating the media being tested as a test substance.
5. Analyze 96-well plate as described in **Section 12.0,** comparing the data from the DMSO controls made using previously tested tissue culture media to the new media being tested.
6. Use the agonist historical database to determine if the new media with DMSO lies within 2.5 standard deviations of the mean for the media. If the RLU values for the new media with DMSO lie within 2.5 standard deviation of the mean for the historical data on DMSO, the new lot of media is acceptable. If the RLU values for the new media with DMSO do not lie within 2.5 standard deviations of the DMSO mean from historical database, the new lot may not be used in the assay.
7. Note date and lot number in study notebook.
8. If the new bottle passes quality testing as described in **Section 15.1 step 6**, apply the media to a single flask of cells and observe cell growth and morphology over the following 2 – 3 days. If there is no change in growth or morphology, the new media is acceptable for use.

15.2 G418:

1. New lots of G418 must first be tested on the LUMI-CELL® ER assay prior to being used in any GLP acceptable assays.
2. Add 220 µL of G418 (previously tested) to a single flask containing cells growing in RPMI.
3. Add 220 µL of G418 (to be tested) to a different flask containing cells growing in RPMI.
4. Observe cellular growth and morphology in both tissue culture flasks over a 48 to 72 hour period. If there are no differences in observed growth rate and morphology between the two flasks, the new G418 lot is acceptable.
5. If cellular growth is decreased, or the cells exhibit abnormal morphology, the new lot of G418 is not acceptable.
6. Note date and lot number in study book.

15.3 DMSO

1. Every new bottle of DMSO must be tested on the LUMI-CELL® ER assay prior to use in any GLP acceptable assays.
2. Add 4 µL of DMSO (to be tested) into four separate 13 mm tubes.
3. Add 400 µL media (previously tested) to the same tubes.
4. Dose an experimental plate as in **Section 12.0,** treating the DMSO containing media being tested as a test substance.
5. Analyze 96-well plate as described in **Section 12.0,** comparing the data from the DMSO controls made using previously tested tissue culture media.
6. Use the agonist historical database to determine if media with new DMSO lies within 2.5 standard deviations of the DMSO mean from historical database. If the RLU values for the media with new DMSO lie within 2.5 standard deviations of the DMSO mean from the historical database, the new lot of DMSO is acceptable. If the RLU values for media with new DMSO do not lie within 2.5 standard

deviations of the DMSO mean from historical database, the new lot may not be used in the assay.

7. Note the date, lot number, and bottle number in study book.

8. If no DMSO has been previously tested, test several bottles as described in **Section 15.3**, and determine whether any of the bottles of DMSO have a lower average RLU than the other bottle(s) tested. Use the DMSO with the lowest average RLU for official experiments.

15.4 Plastic Tissue Culture Materials

1. Grow one set of cells, plate them for experiments on plastic ware from the new lot and one set of cells in the plastic ware from a previous lot, and dose them with E2 reference standard and controls.

2. Perform the LUMI-CELL® ER experiment with both sets of cells.

3. If all of the analysis falls within acceptable QC criteria, then the new manufacturer's products may be used.

16.0 REFERENCES

Eli Lilly and Company and National Institutes of Health Chemical Genomics Center. 2005. Assay Guidance Manual Version 4.1. Bethesda, MD: National Institutes of Health. Available: http://www.ncgc.nih.gov/guidance/manual_toc.html [accessed 05 September 2006]

ICCVAM. 2001. Guidance Document on Using *In Vitro* Data to Estimate *In Vivo* Starting Doses for Acute Toxicity. NIH Pub. No. 01-4500. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/invidocs/guidance/iv_guide.pdf [accessed 31 August 2006]